Evaluation of the antimicrobial activity of the K9CATH peptides of 21 and 38 amino acids against *Brucella abortus* and *Brucella melitensis*

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Abstract

Brucellosis is a worldwide zoonotic disease endemic in Mexico and of major economic importance to the livestock industry and public health. The increase of resistance and reduced susceptibility to common anti-brucella drugs in several Brucella species reported in recent studies have motivated the researchers to search for new antimicrobials where the antimicrobial peptides can be an alternative. The present is a pilot study in which the in vitro antimicrobial susceptibility of two bovine field strains of *B. abortus* and *B. melitensis* were evaluated against the antimicrobial peptides canine cathelicidin (K9CATH) of 21 and 38 amino acids. Minimum inhibitory concentration (MIC) of 8 µg/ml for both cathelicidins was obtained against *B. abortus*, while none of the cathelicidins up to 128 µg/ml inhibited *B. melitensis* growth. Further studies including more clinical isolates of *brucella* and the use of combinations of these peptides with the conventionally prescribed anti brucellosis drugs could be useful in ascertaining whether these peptides have a synergistic effect.

Introduction

Brucellosis is one of the main zoonosis of bacterial origin with a global impact on both public and animal health, generating great economic loss in the livestock industry. The causative agents are bacteria of the genus *Brucella* which comprises 10 recognized species of which *Brucella abortus*, *Brucella melitensis* and *Brucella suis* are the three main zoonotic species (Trott *et al.*, 2018). Brucelosis in Mexico is an endemic and notifiable disease both in humans and animals and, despite the existence of a control and eradication program, annual economic loss of up to 90 million US dollars has been estimated due to positive cases of bovine brucellosis (Luna-Martínez and Mejía-Terán, 2002). In 2006 human brucelosis ranked twenty-first in the world and second in the Americas with an incidence rate of 2.00 to 2.64 per 100,000 inhabitants (Guzmán-Hernández *et al.*, 2016; Guzmán-Bracho *et al.*, 2020), mainly due to the consumption of contaminated fresh cheese and unpasteurized dairy products (Luna-Martínez and Mejía-Terán, 2002; Méndez-Lozano *et al.*, 2015; Guzmán-Hernández *et al.*, 2016). *Brucella* is an intracellular pathogen that requires long treatment with antibiotics that may efficiently penetrate the cells; therefore, availability of effective antibiotics against this microorganism are limited. The treatment regimen recommended by the World Health Organization (WHO) includes a combination of different antibiotics such as doxycycline, rifampicin, tetracycline, streptomycin, trimethoprim-sulfamethoxazole and for years no major changes in treatment schedules have been made (WHO 1986; Heo *et al.*, 2012). Susceptibility to these antibiotics may change over time and from one geographical region to another. Moreover, in vitro susceptibility tests are not standardized for the *Brucella* species and they are not routinely performed. Nevertheless there is enough evidence from antimicrobial susceptibility studies performed with a variety of antimicrobial agents and different strains of *Brucella* isolated from animals to show some degree of resistance (Bayram *et al.*, 2011; Maves *et al.*, 2011; Barbosa Pauletti *et al.*, 2015). Some of the anti-brucella drugs are also used to treat tuberculosis (Baykam *et al.*, 2004; López-Merino *et al.*, 2004) in places such as Mexico where both diseases are endemic (Trott *et al.*, 2018) and this may have led to a reduction in the susceptibility of the organisms or an increased possibility of resistance developing. Therefore, it is necessary to evaluate alternative antimicrobial agents, such as antimicrobial peptides (AMP´s). The AMP´s are well-characterized essential components of the innate immune system conserved throughout evolution with a broad antimicrobial spectrum against intracellular microorganisms and they are non-toxic to the host (Linde *et al.*, 200; Bahar and Ren, 2004; Kalita *et al.*, 2004). Specifically, cathelicidins are a group of cationic peptides present in leukocytes and epithelial cells that play an important role in early innate immune responses against infections. The K9CATCH peptide is a dog cathelicidin present in the content of neutrophil granules and synthetic K9CATH has shown extensive antimicrobial activity in vitro against gram positive and gram negative bacteria and yeast (Sang *et al.*, 2007). Furthermore, a study carried out by Tamayo

et al., (2012) showed the antimicrobial effect of K9CATH in vitro against *Mycobacterium tuberculosis* H37Rv at a concentration of 10.66 µg/ml and, in vivo, during the treatment of mice with induced pulmonary tuberculosis with the same strain at a concentration of 32 µg/ml. In addition the K9CATH peptide has shown to be effective against *Staphyloccocus aureus* in vitro (MIC 5.66 µg/ml) and during the treatment of experimental mastitis in mice reducing significantly the colony-forming units at a concentration of 32 µg/ml (Barreras-Serrano *et al.*, 2012; Barreras-Serrano *et al.*, 2014; Barreras-Serrano *et al.*, 2017). Since *Brucella*, *Mycobacterium* and *Staphyloccocus* are intracellular pathogens it has been considered relevant to evaluate the antimicrobial activity of synthetic K9CATH peptides of 21 and 38 amino acids in cultures of *B. abortus* and *B. melitensis*.

Materials and Methods

Preparation of bacterial inoculum: Two field strains, one of *B. abortus* and one of *B. melitensis*, were cultured in Trypticase soy agar (Becton Dickinson, France) added with 5% bovine serum and incubated for 48 hours at 37°C at 5% CO_2. A bacterial suspension was prepared to equal tube No. 1 on the McFarland scale, corresponding to 300 ×10⁶ bacteria/ml, from which a 1:20 dilution was prepared to be used in a 96-well microplate.

Preparation of antimicrobial peptide K9CATH: The K9CATH antimicrobial peptides of 21 and 38 amino acids were donated by Dr. Melgarejo of the Western University of Health Sciences. The 38 aa´s K9CATH peptide (RLKELITTGGQKIGEKIRRIGQRIKDFFKNLQPREEKS) was the originally discovered and synthetized peptide with antimicrobial properties reported against a variety of bacteria (Sang *et al.*, 2007). The 21 amino acid peptide (sequence under registration process) originated from a modification of the 38 amino acid peptide, where 17 amino acids were removed to obtain a shorter length peptide (a feature that is directly related to synthesis production costs), in order to improve such features as antimicrobial potential, nontoxicity and non-susceptibility to protease cleavage to make it more suitable for therapeutic application (unpublished data). However the antimicrobial effect of these peptides against *Brucella* is unknown. Each peptide was supplied in individual vials of 2 mg of lyophilized synthetic antimicrobial peptide K9CATH. Each peptide was reconstituted in 500 µL of sterile deionized water obtaining a final concentration of 4 µg/µL. For the assay a stock solution at a concentration of 512 µg/ml for each of the 21 and 38 amino acid K9CATH peptides was prepared.

Resazurin Microplate Method (RMM): Minimum inhibitory concentration (MIC) for the K9CATH was determined following the protocol described by Franzblau *et al.*, (1998) with minor modifications. Briefly, 100 µl of Mueller-Hinton II broth was added to a 96-well plate (Falcon 3072; Becton Dickinson, Lincoln Park, N.J.), following the addition of stock solution of the 21 amino acid peptide to half of the 96 well plate

and stock solution of the 38 amino acid peptide to the other half and, from those, serial dilutions downwards were made to obtain final concentrations of 128, 64, 32, 16, 8 and 4 μg/ml of each peptide. Then 100 μl of bacterial suspension of *B. abortus* and *B. melitensis* was added to the wells containing the peptides. Also, a viability control for *B. abortus* and *B. melitensis* was included consisting of Mueller-Hinton II medium and the bacterial suspension. Also a control of sterility was included containing only Mueller-Hinton II medium. The plate was incubated for 48 hours at 37°C and 5% CO_2. After that, 30 μl of 0.1% resazurin solution were added to all the wells and incubated for an additional 24 hours. The assay was performed in triplicate for each peptide concentration for each bacteria strain. The MIC values corresponded to the last well that remained blue. A blue color in the well was an indicator of non-bacterial growth, while the development of a pink color was an indicator of bacterial growth.

Results and Discussion

The 21 and 38 amino acid K9CATH peptides inhibited *B. abortus* growth with a MIC of 8 μg/ml, while no inhibition was observed for *B. melitensis* to a concentration up to 128 μg/ml with any of the peptides (Table 1). The lack of effectiveness of the K9CATH peptides on the strain of *B. melitensis* from this study could have been due to differences in the outer membrane (OM) composition, since it has been shown that the lipopolysaccharide plays a role in the resistance of *Brucella spp* to polycationic compounds both at the O chain and core lipid A, as suggested by Martinez de Tejada *et al.*, (1995) in a study where the resistance of *B. abortus*, *B. melitensis* and *B. ovis* species to 14 bactericidal cationic peptides was studied.

Table 1 Minimal inhibitory concentration (MIC) for the K9CATH antimicrobial peptides of 21 and 38 amino acid against *B. abortus* was 8 μg/ml, and for *B. melitensis* no inhibition was observed with any of the peptides.

K9CATH 21 aa´s	*Brucella abortus*	*Brucella melitensis*
128 μg/ml	− blue	+ pink
64 μg/ml	− blue	+ pink
32 μg/ml	− blue	+ pink
16 μg/ml	− blue	+ pink
8 μg/ml	**MIC**	+ pink
4 μg/ml	+ pink	+ pink

K9CATH 38 aa´s	*Brucella abortus*	*Brucella melitensis*
128 μg/ml	− blue	+ pink
64 μg/ml	− blue	+ pink
32 μg/ml	− blue	+ pink
16 μg/ml	− blue	+ pink
8 μg/ml	**MIC**	+ pink
4 μg/ml	+ pink	+ pink

− No bacterial growth = blue color; + Bacterial growth = pink color

Brucella abortus and *B. melitesis* are the main causative agents of brucellosis in both humans and animals in Mexico. For an effective treatment against brucellosis it is important to consider three main aspects: the use of antimicrobials capable of penetrating the cells, the use of combined antimicrobials rather than single compounds and an evaluation of in vitro susceptibility of these antimicrobials (Bahar and Ren, 2013). However, in Mexico, *Brucella* species involved in clinical cases are not usually identified and neither are susceptibility assays performed in vitro, with treatments based on protocols established by the Secretary of Health using combinations of tetracycline, streptomycin, rifampin, trimethoprim-sulfamethoxasole and doxycycline (NOM-022-SSA2-2012). Furthermore, Mexico has high rates of both brucellosis and tuberculosis and the use of certain agents such as rifampicin is a common treatment for both diseases, so prolonged administration of rifampicin for brucellosis may increase the resistance of *M. tuberculosis* to this compound or vice versa (Trott *et al.*, 2020; Mohammadi

et al., 2017). Moreover, experimental data suggests that the development of mycobacterial resistance to rifampicin may lead to the development of resistance to other antimicrobials as well (Araiza *et al.*, 2007). Consequently it is necessary to examine new antimicrobials that are capable of penetrating the cells, hence the AMPs can be an alternative and specifically the K9CATH peptides which have proven to be effective against *M. tuberculosis* both in vitro (MIC 10.66 μg/ml) and in vivo during the treatment of pulmonary tuberculosis in mice (Barreras-Serrano *et al.*, 2014), as well as against *S. aureus* in vitro (MIC 5.66 μg/ml), and during the treatment of clinical mastitis induced in mice (at 32μg/ml). The effectiveness of the antimicrobial peptides against some *Brucella* species has been demonstrated by a previous study by Andrä *et al.*, (2007) that showed the antimicrobial activity of the peptides NK-2, NK27, C20-DK, NK23c, NK19b, NK19b-KR, and melittin against *B. abortus*. Also a study by Mohammadi *et al.*, (2017), showed the in vitro synergistic and additive antimicrobial effect of a short cationic peptide (CM11) when combined with

clinically used antibiotics against drug-resistant isolates of *B. melitensis*. The results in this pilot study indicate that *B. abortus* is susceptible to the 21 and 38 amino acid K9CATH peptides whereas *B. melitensis* is not. The MIC for the K9CATH peptides against *B. abortus* obtained in this study falls within the concentration range obtained with the two previous intracellular pathogens evaluated. In this study the MIC combination assay was not included and therefore the MIC of antibiotics in combination with these K9CATH peptides was not assessed and only the K9CATH peptide MIC was determined. As a pilot study the purpose was to generate preliminary data on the antimicrobial effect that these two peptides could have against the two most endemic *Brucella* species in Mexico for further formal study. However, further studies are needed to fully understand the lack of effectiveness of these peptides against *B. melitensis*. Also it is necessary to include a larger number of *Brucella* strains collected from different endemic areas and since antimicrobial peptides can be used in synergy with conventional antibiotics (Azad *et al.*, 2017), it is suggested that K9CATH be evaluated in combination with the main antimicrobials used to treat brucellosis.

Acknowledgement

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